Commercial Ripening Starter Microorganisms Inoculated into Cheese Milk Do Not Successfully Establish Themselves in the Resident Microbial Ripening Consortia of a South German Red Smear Cheese[∇]

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Production of smear-ripened cheese critically depends on the surface growth of multispecies microbial consortia comprising bacteria and yeasts. These microorganisms often originate from the cheese-making facility and, over many years, have developed into rather stable, dairy-specific associations. While commercial smear starters are frequently used, it is unclear to what degree these are able to establish successfully within the resident microbial consortia. Thus, the fate of the smear starters of a German Limburger cheese subjected to the "old-young" smearing technique was investigated during ripening. The cheese milk was supplemented with a commercial smear starter culture containing *Debaryomyces hansenii*, *Galactomyces geotrichum*, *Arthrobacter arilaitensis*, and *Brevibacterium aurantiacum*. Additionally, the cheese surface was inoculated with an extremely stable in-house microbial consortium. A total of 1,114 yeast and 1,201 bacterial isolates were identified and differentiated by Fourier transform infrared spectroscopy. Furthermore, mitochondrial DNA restriction fragment length polymorphism, random amplified polymorphic DNA, repetitive PCR, and pulsed field gel electrophoresis analyses were used to type selected isolates below the species level. The *D. hansenii* starter strain was primarily found early in the ripening process. The *G. geotrichum* starter strain in particular established itself after relocation to a new ripening room. Otherwise, it occurred at low frequencies. The bacterial smear starters could not be reisolated from the cheese surface at all. It is concluded that none of the smear starter strains were able to compete significantly and in a stable fashion against the resident microbial consortia, a result which might have been linked to the method of application. This finding raises the issue of whether addition of starter microorganisms during production of this type of cheese is actually necessary.

The ripening of red smear cheeses depends on the growth on their surfaces of microbial consortia consisting of yeasts and coryneform bacteria and other gram-positive bacteria such as staphylococci (7, 9, 19, 34). The compositions of these consortia differ significantly between different cheeses and manufacturers (13, 22, 24) and can be quite complex and are often unknown (30). It has been shown that the species composition of such surface consortia can be remarkably constant over time (22), and it is known that it can be largely influenced by a well-established individual house microflora (1, 25). To ensure the production of a high-quality cheese, the so-called old-young smearing technique is often used. During this traditional process, a smear of ripe ("old") cheeses is washed off their surfaces to inoculate freshly produced ("young") cheese (9, 34). All microorganisms that develop during and contribute to the ripening process are transferred to the smear brine and thereby to the surface of the young cheese. The surface microbial species are well adapted to biotic and abiotic factors of the cheese environment. This leads to fast development of the

characteristic microbial consortia and, therefore, to an optimal ripening process (1, 3, 5).

On the other hand, the old-young smearing process also leads to reinfection of freshly produced cheese by undesired contaminants of the ripe cheese, most importantly by pathogens such as *Listeria monocytogenes* (6, 9, 22, 34). It is, therefore, an important task to develop complex, defined ripening cultures which can be added to the fresh cheeses and eventually may completely replace the old-young smearing process (1, 3, 37). However, limited knowledge about the microbial ecology of these cheeses makes the development of commercially available, defined smear cultures difficult, since optimal ripening conditions and cheese quality have to be guaranteed (1). To enhance or support the ripening process induced by the old-young smearing technique, cheese manufacturers often additionally supplement the cheese milk or smear brine with commercial, defined smear starter strains. It is expected that such an action eventually might establish defined microbial consortia consisting predominantly of these defined starter microbes. However, very little is known about the development of the defined starter organisms on the cheese surface. Will these microbes be able to establish in the presence of the resident consortia during a single ripening period? If not, will the continued addition of starters over many production cycles help to establish a novel consortium? There are only limited data

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scattered in the literature indicating that starter microorganisms do not easily compete with the resident, undefined microbial consortia (8, 14, 24, 28).

To study the fate of commercial smear starter strains, a detailed and quantitative analysis of the species and strain composition at different times during cheese ripening is necessary. To this end, we have applied Fourier transform infrared (FTIR) spectroscopy as a rapid and cost-efficient tool to differentiate and identify hundreds of bacterial and yeast isolates (20, 26, 27). For typing below the species level, several genotypic methods have been applied. To our knowledge, this is the first comprehensive and systematic study of the fate of commercial smear starter microorganisms during the development of a resident cheese surface microbial consortium.

MATERIALS AND METHODS

Production of Limburger cheese. The Limburger cheese investigated in this study was produced in a German dairy using pasteurized milk and a thermophilic lactic acid starter culture. Two days after production the cheeses were surface smeared the first time (day 2); this was followed by two to three further smear treatments every 2 to 3 days, depending on the progression of ripening. A modified old-young smearing technique was used in which the first smearing of a batch followed the second smearing of the previously produced batch. This process continued with the third and fourth smearings of older batches. Furthermore, a commercial smear starter culture (PLA 50 D CHOOZIT cheese cultures; DANISCO, France [formerly Rhodia Food, France]) was added to the milk before renneting, with an inoculation level of 1 dose per 1,000 liters (2.0×10^{10} to 2.6×10^{11} CFU/dose) as suggested by the culture supplier. Since no information from the culture supplier was made available about cell numbers of individual species in the culture, we performed our own studies of this culture, resulting in determinations of 1.3×10^{10} to 2.3×10^{10} CFU/dose in three independent experiments, with 1.2×10^7 to 2.6×10^7 CFU/dose of *Debaryomyces hansenii*, 2.6×10^7 to 4.6×10^7 CFU/dose of *Geotrichum candidum*, 2.1×10^9 to 7.5×10^9 CFU/dose of *Brevibacterium linens*, and 1.1×10^{10} to 1.5×10^{10} CFU/dose of *Arthrobacter nicotianae*. After 14 days, cheeses were packaged for sale with an expiration date set to about 60 days after production.

Isolation of microorganisms from the surface of Limburger cheese. Eight independently produced Limburger batches were investigated for the presence of the commercial yeast and bacterial smear starter microorganisms on the cheese surface during several ripening stages. The first three batches were produced during January and April 2002, and a further production batch was sampled in December 2004. The other four batches, produced between April and July 2006, were studied to record the relocation from the old ripening room (batches 1 to 6) to a new ripening room (batches 7 and 8) (Table 1). The new ripening room was disinfected once per week, and process parameters were automatically controlled. For storage, and to ensure the status of ripening to be investigated, the cheeses were frozen at −70°C. The surface microflora was studied after 6, 28, and 58 days (batches 1 to 3) and, to focus especially on the early ripening time when most flora changes were found, after 3, 6, 9, and 28 days (batch 4 to 8) of ripening. A total of 50 cm² of cheese surface was cut with a sterile knife to about 2 mm in depth, diluted 10-fold with trisodium citrate buffer (2 g/100 ml; pH 7.5), and homogenized. A total of 1,114 yeast and 1,201 coryneform isolates were collected from plates with countable dilutions plated. Yeasts were grown on yeast extract glucose chloramphenicol agar (Merck, Darmstadt, Germany) supplemented with 10 mg/liter bromophenol blue (YGCBA) (33) and incubated for 5 days at 20°C. Isolation of bacteria was generally focused on coryneforms and gram-positive, catalase-positive cocci. Bacteria of batches 1 to 3 were cultivated on plate count agar containing 3% NaCl (PCA³⁺; 5.0 g tryptone [Merck], 2.5 g yeast extract [Oxoid, Basingstoke, England], 1.0 g glucose [Fluka/Sigma-Aldrich, Steinheim, Germany], 30.0 g NaCl [Roth, Karlsruhe, Germany], 15.0 g agar [Oxoid], and 1 liter distilled water; adjusted to pH 7.0 ± 0.2). Isolates were collected after 5 days of incubation at 20°C under aerobic conditions. Since experience showed that several coryneform bacteria grew better on tryptic soy agar (TSA; Roth, Karlsruhe, Germany) than on PCA³⁺, both media were used in the fourth batch of cheese. TSA plates were incubated at 30°C for 3 days before isolation. Bacteria of batches 5 to 8 were isolated from CRBM (11) after 5 days at 20°C. To isolate bacteria, development of yeast and fungi was suppressed by spreading 100 μl of 2% pimaricin (8) (Sigma-Aldrich; approximately 2.5% aqueous suspension) on the surface of each PCA³⁺, TSA, or CRBM

TABLE 1. Fractions of *D. hansenii* and *G. geotrichum* strains found within eight independently produced Limburger batches during ripening

Mo/yr of production (batch no.)	No. of days of ripening	% strain in batch (total no. of isolates)[a]			
		D. hansenii		*G. geotrichum*	
		Starter strain	Non-starter strain	Starter strain	Non-starter strain
1/2002 (1)	6	14 (7)	74 (37)	—[c]	12 (6)
	28	2 (1)	78 (39)	—	20 (10)
	58	12 (6)	66 (33)	—	2 (11)
2/2002 (2)	6	4 (2)	37 (18)	—	9 (29)
	28	—	75 (44)	—	5 (15)
	58	4 (2)	78 (38)	—	8 (9)
4/2002 (3)	6	—	95 (52)	—	5 (3)
	28	—	90 (46)	—	10 (5)
	58	5 (3)	79 (44)	—	16 (9)
12/2004 (4)	3	88 (44)	—	12 (6)	—
	6	16 (8)	—	6 (3)	78 (39)
	9	15 (3)	—	5 (3)	70 (14)
	28	18 (9)	—	20 (10)	62 (31)
4/2006 (5)[b]	3	67 (20)	—	20 (6)	3 (1)
	6	3 (1)	—	13 (4)	73 (22)
	9	7 (2)	—	13 (4)	73 (22)
	28	10 (3)	—	30 (9)	60 (18)
4/2006 (6)[b]	3	20 (6)	—	73 (22)	—
	6	—	—	30 (9)	70 (21)
	9	3 (1)	—	7 (2)	87 (26)
	28	3 (1)	—	23 (7)	73 (22)
6/2006 (7)[b]	3	37 (11)	—	47 (14)	3 (1)
	6	3 (1)	—	10 (3)	80 (24)
	9	20 (6)	—	63 (19)	17 (5)
	28	13 (4)	—	57 (17)	27 (8)
7/2006 (8)[b]	3	16 (4)	—	12 (3)	—
	6	3 (1)	—	10 (3)	83 (25)
	9	—	—	13 (4)	83 (25)
	28	10 (3)	—	27 (8)	63 (19)

[a] Absolute numbers in parentheses refer to the total number of isolates within each ripening stage.

[b] Fractions refer to total numbers of isolates for the individual ripening stages of the indicated batches, yielding 30 each, except the day 3 sample of batch 8, which yielded 25 isolates. Differences between total numbers of isolates for the individual ripening stages of the indicated batches correspond to numbers of isolates determined to be yeast species other than *D. hansenii* and *G. geotrichum*.

[c] —, strain not detectable at this sampling point.

plate before use. Yeasts and bacteria were subcultured on YGCBA and PCA³⁺ lacking pimaricin, respectively. The isolates were stored at −80°C as glycerol cultures. To this end, fresh cell material grown on YGCBA or PCA³⁺ for yeast or bacteria, respectively, was suspended in 5 ml of suspension medium (10.0 g sodium glutamate [AppliChem, Darmstadt, Germany], 16.0 g lactose [Merck], 1.0 g agar [Oxoid], 0.1 g ascorbic acid [Merck], 120.0 g glycerol [Merck], and 1 liter of tap water).

Isolation of microorganisms from a commercial smear starter culture. To isolate the microorganisms from the freeze-dried commercial smear starter mixed culture used to produce the Limburger cheese, one spatula of powder was added to 1 ml of yeast extract glucose broth (5.0 g yeast extract [Oxoid], 20.0 g glucose [Fluka, Sigma-Aldrich], and 1.0 liter of distilled water; pH 6.6 adjusted with HCl, autoclaving at 121°C for 15 min). The suspension was held at a refrigeration temperature for around 2 h to give the organisms time to resuscitate. Then, aliquots of appropriate dilutions were plated in duplicate on YGCBA for yeast isolation or on PCA³⁺ with 100 μl 2% pimaricin spread onto the agar surface for collecting bacteria. In 2002, only one representative of each of the four culture organisms was available. The possibility cannot be excluded that different strains of the same species may be present in a starter culture and, subsequently, may establish differently in the surface consortia. To check for potential intraspecies variability of the starter, in 2004, a random sample of 50 colonies of each of the four ripening starter culture organisms was taken accord-

ing to their colony morphologies and studied by FTIR spectroscopy. Representative strains were subjected to further analyses.

FTIR spectroscopy. Sample preparation, measurement, and evaluation were performed according to Kümmerle et al. (20) for the yeasts and according to Oberreuter et al. (27) for the coryneform bacteria. An IFS-28B FTIR spectrometer was used with OPUS 3.17 software for Windows (both from Bruker, Karlsruhe, Germany). The reference databases, maintained at the Technical University of Munich, comprised about 2,500 spectra of type and reference strains of relevant species for the identification of yeasts and approximately 1,200 spectra for the identification of coryneform bacteria and staphylococci.

mtDNA RFLP analysis. Mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) for typing the yeasts was carried out as described by Mounier et al. (24). Purified DNA was digested with HaeIII. Similarities among band patterns were calculated based on the Dice similarity coefficient and the unweighted-pair group method using average linkages (UPGMA) algorithm.

RAPD analysis. DNA extraction followed the same protocol as described for mtDNA RFLP analysis (24). Random amplified polymorphic DNA (RAPD) analysis was performed on isolates assigned to *G. geotrichum* following the description of Brennan et al. (8) and using the M13 forward primer (5'-GTAA AACGACGGCCAGT-3'). For similarity analyses of band patterns, the Dice similarity coefficient and the UPGMA algorithm were used.

PFGE analysis. Pulsed-field gel electrophoresis (PFGE) analysis of coryneform bacteria was performed according to the protocol published by Brennan et al. (8). Chromosomal DNA was digested using AscI, SpeI, or XbaI enzyme.

***rep*-PCR.** Repetitive PCR (*rep*-PCR) was performed using coryneform bacteria and BOXA1R primers (35), with DNA extracted according to the method of Gevers et al. (16). Pearson's similarity coefficient and the UPGMA algorithm were used to analyze the resulting band patterns. Individual isolates from *rep*-PCR clusters were subjected to 16S rRNA gene sequence analysis for identification, using primers described by Coenye et al. (10). Genomic DNA obtained for the *rep*-PCR was also used for the 16S rRNA gene sequence analyses.

RESULTS

Viable counts of yeast and bacteria. The yeast numbers ranged from 7×10^3 to 10^4 CFU/cm^2 at day 3 and up to 10^5 to 10^7 CFU/cm^2 at days 28 and 58. The bacterial cell counts increased from 5×10^3 to 5×10^4 CFU/cm^2 at day 3 up to 10^7 to 10^8 CFU/cm^2 at day 28, remaining at this level until day 58.

Identification and differentiation of yeasts and bacteria by FTIR spectroscopy. Eight Limburger batches were examined for the incidence of the commercial smear starters on their surfaces during different ripening stages. A total of 1,114 yeast and 1,201 bacterial isolates were collected. All yeast isolates of batches 1 to 4 and the predominant parts of batches 5 to 8 were identified as *D. hansenii* or *G. geotrichum* by FTIR spectroscopy (Table 1). Rarely, *Issatchenkia orientalis*, *Kluyveromyces marxianus*, *Yarrowia lipolytica*, and *Candida* spp. were found in the last four batches. Each of the two dominant species occurred in two different colony morphologies when cultivated on YGCBA. *D. hansenii* type A, which only occurred in batches 1 to 3, was characterized by a light blue, matt, and rough colony surface, while *D. hansenii* type B, which occurred in all eight batches, was shiny, with a bright blue surface surrounded by a white edge (see pictures in Fig. 3). *G. geotrichum* type 1 showed a turquoise- to blue-colored colony surface, and *G. geotrichum* type 2 had a light green and fluffy appearance (see pictures in Fig. 4). Whereas *G. geotrichum* type 1 was found in all batches, type 2 was detected in batches 4 to 8. The coryneform isolates of all eight Limburger batches were assigned to either *A. arilaitensis* or *B. aurantiacum*, two newly described species (15, 18). Occasionally, gram-negative bacteria and bacilli were detected; these, however, were not in the focus of the study.

FTIR spectra of the yeast and bacterial surface isolates were compared with those of the respective smear starter represen-
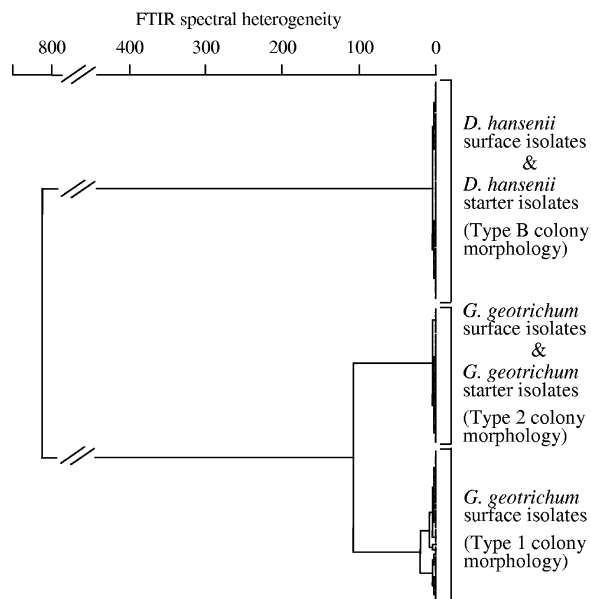

FIG. 1. Dendrogram depicting FTIR spectral heterogeneities between the yeast surface isolates and the yeast starters in batch 4. The colony morphology found for *D. hansenii* on YGCBA was type B (colonies with a bright blue center surrounded by a white edge); colony morphologies found for *G. geotrichum* were type 1 (turquoise- to blue-colored colony surface) and type 2 (light green with a fluffy colony surface). The dendrogram was constructed using Ward's algorithm and correlation with normalization to reproducibility level. The second derivatives of spectra were used. Frequency ranges were 3,030 cm^{-1} to 2,830 cm^{-1}, 1,350 cm^{-1} to 1,200 cm^{-1}, and 900 cm^{-1} to 700 cm^{-1}. Each frequency range has a weight value of 1 and a reproducibility level of 30, where the weight value represents the importance given to each frequency range and the reproducibility level is used for adjusting the spectral distance.

tatives by use of cluster analysis. Representatives of the *D. hansenii* starter (type B colony morphology) showed high spectral similarity to 88% (batch 4), 67% (batch 5), and 37% (batch 7) of the yeast isolates collected from the cheese samples obtained after the first smearing. However, high similarity to the isolates in all the other ripening stages of all eight batches was only found for 0 to 20% of the isolates. No spectral similarity was observed between representatives of the *G. geotrichum* starter and surface isolates obtained from batches 1 to 3, but 5 to 20% of the spectra of batch 4 isolates clustered together with those of the *G. geotrichum* starter. With the exception of the early stage of the sixth batch, similar or only marginally higher results were obtained with batches 5 and 6. Directly after the relocation to the new cellar, for batch 7, the *G. geotrichum* starter strain showed significantly increased fractions of 47 to 63%. However, in the last batch studied those results were again decreased to a level comparable to those seen with the other batches. Most of the *G. geotrichum* isolates from the eight batches showed the type 1 colony morphology when cultivated on YGCBA, whereas the minority of the *G. geotrichum* isolates, including the fraction that showed similarity to the *G. geotrichum* starter as well as the starter representatives, were of type 2 colony morphology (Table 1). To illustrate this result, a dendrogram depicting the FTIR spectral similarity of the yeast surface isolates from batch 4 and the commercial yeast starters is presented in Fig. 1. Spectra of both

TABLE 2. Fractions of *A. arilaitensis* and *B. aurantiacum* strains found within eight independently produced Limburger batches during ripening

Mo/yr of production (batch no.)	No. of days of ripening	% strain in batch (total no. of isolates)[a]			
		A. arilaitensis		*B. aurantiacum*	
		Starter strain	Non-starter strain	Starter strain	Non-starter strain
1/2002 (1)	6	—[d]	—	—	100 (39)
	28	—	57 (25)	—	43 (19)
	58	—	76 (34)	—	24 (11)
2/2002 (2)	6	—	24 (11)	—	76 (34)
	28	—	74 (37)	—	26 (13)
	58	—	70 (33)	—	30 (14)
4/2002 (3)	6	—	74 (34)	—	26 (12)
	28	—	84 (41)	—	16 (8)
	58	—	71 (35)	—	29 (14)
12/2004 (4)	3	ND[c]	ND	ND	ND
	6	—	45 (34)	—	55 (41)
	9	—	81 (131)	—	19 (30)
	28	—	93 (70)	—	7 (5)
4/2006 (5)[b]	3	—	83 (25)	—	13 (4)
	6	—	73 (22)	—	27 (8)
	9	—	100 (30)	—	
	28	—	90 (27)	—	3 (1)
4/2006 (6)[b]	3	—	90 (27)	—	10 (3)
	6	—	37 (11)	—	63 (19)
	9	—	100 (30)	—	—
	28	—	83 (25)	—	17 (5)
6/2006 (7)[b]	3	—	90 (27)	—	7 (2)
	6	—	77 (23)	—	17 (5)
	9	—	39 (28)	—	7 (2)
	28	—	57 (17)	—	36 (11)
7/2006 (8)[b]	3	—	90 (27)	—	7 (2)
	6	—	57 (17)	—	33 (10)
	9	—	93 (28)	—	3 (1)
	28	—	77 (20)	—	23 (6)

[a] Absolute numbers in parentheses refer to the total number of isolates within each ripening stage.

[b] Fractions refer to total numbers of isolates for the individual ripening stages of the indicated batches, yielding 30 each, except the day 28 sample of batch 8, which yielded 26 isolates. Differences between total numbers of isolates for the individual ripening stages of the indicated batches correspond to numbers of isolates determined to be bacterial species other than *A. arilaitensis* and *B. aurantiacum*.

[c] ND, not determined.

[d] —, strain not detectable at this sampling point.

bacterial starters did not show any similarity to those of the surface isolates from all batches (Table 2), as seen in Fig. 2. It is noteworthy that *A. arilaitensis* (batches 1 to 8) and *B. aurantiacum* (batches 5 to 8) surface isolates clearly formed different spectral groups within each species, as shown for *A. arilaitensis* in Fig. 2.

Typing of yeasts by mtDNA RFLP. For yeast typing, a total of 49 isolates assigned to *D. hansenii* and *G. geotrichum* from the first four cheese batches were selected based on FTIR clusters and subjected to mitochondrial DNA RFLP analyses. In addition, representative isolates of the *D. hansenii* and *G. geotrichum* starter strains used in 2002 and 2004 were analyzed. Two different profiles were detected for *D. hansenii*. Both profiles were present within surface isolates of batches 1 to 3, whereas only one of these profiles could be assigned to batch 4 surface isolates (Fig. 3). The latter profile also corresponded to those seen with the smear starter representatives. RFLP analysis of *G. geotrichum* surface

isolates also resulted in two different profiles (Fig. 4). Variations in one or two bands were not considered to be significant enough to indicate different patterns. Surface isolates collected from the first three batches could be assigned to the profile representing type 1 colony morphology, whereas batch 4 isolates comprised both RFLP profiles. The second profile, assigned to type 2 colony morphology, was also determined for the *G. geotrichum* smear starters tested.

Typing of yeasts by RAPD-PCR. In addition to the mtRFLP analysis, RAPD-PCR studies were performed on 21 *G. geotrichum* surface isolates and three smear starter representatives from 2002 and 2004. Two RAPD patterns could be distinguished which corresponded to the different colony morphology types observed for *G. geotrichum*. The first pattern was found for surface isolates from all four batches. The other pattern was found only in surface isolates obtained from batch 4 cheeses as well as in all starter representatives (data not shown).

Typing of bacteria by *rep*-PCR. For studies of the bacterial biodiversity, BOX-PCR was used for typing of the Limburger surface isolates of batches 1 to 4 and was combined with 16S rRNA gene sequence analysis for identification. These methods were also applied to the bacterial smear starters. Both the surface isolates and the smear starters, the latter designated *A. nicotianae* and *B. linens* by the culture supplier, were identified as *A. arilaitensis* or *B. aurantiacum* (15, 18). However, the *rep*-PCR patterns of the starters were different from those of the respective surface isolates (Fig. 5).

Typing of bacteria by PFGE analysis. To further clarify the relationships between the bacterial surface isolates and smear
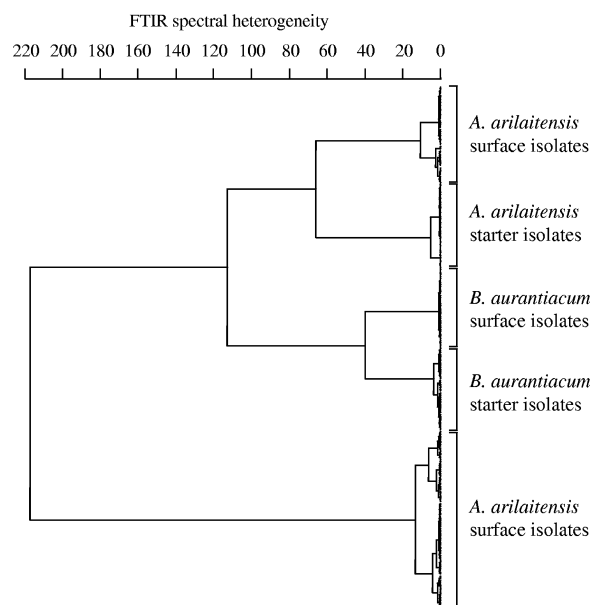

FIG. 2. Dendrogram depicting FTIR spectral heterogeneities between bacterial surface isolates from batch 4 and the ripening starter strains. The dendrogram was constructed using Ward's algorithm and correlation with normalization to reproducibility level. The first derivatives of spectra were used. Frequency ranges with weights and reproducibility levels were 3,000 cm^{-1} to 2,800 cm^{-1} (weight, 0.8; reproducibility level, 3.3), 1,800 cm^{-1} to 1,500 cm^{-1} (0.8 and 5), 1,500 cm^{-1} to 1,200 cm^{-1} (0.9 and 20), 1,200 cm^{-1} to 900 cm^{-1} (0.9 and 33), and 900 cm^{-1} to 700 cm^{-1} (0.9 and 116). See Fig. 1 legend for explanation of weight and reproducibility level data.

Dice (Tol 2.0%-2.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
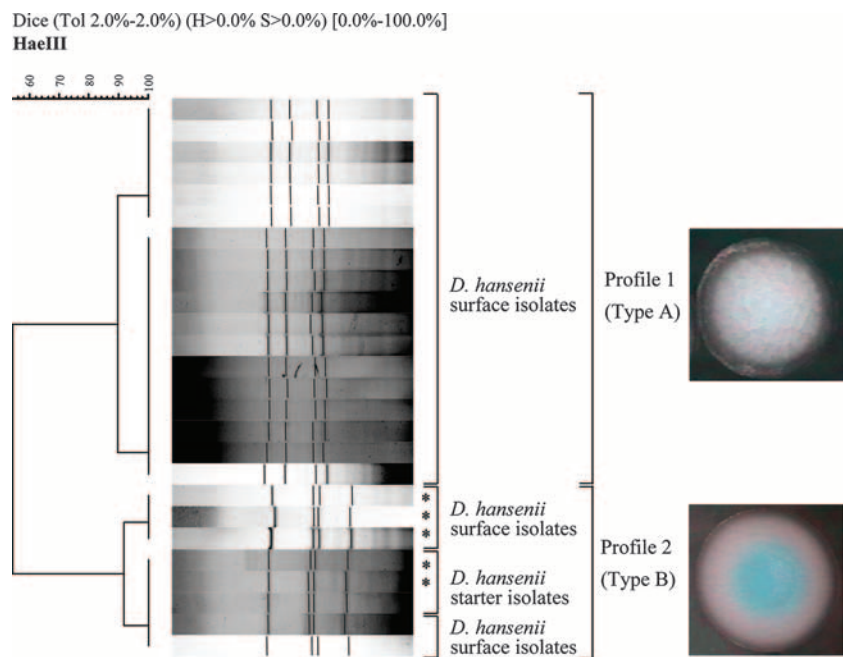**HaeIII**

FIG. 3. Mitochondrial DNA RFLP profiles of *D. hansenii* surface isolates as well as *D. hansenii* starters. Purified DNA was digested with HaeIII. Similarities among band patterns were calculated using the Dice similarity coefficient and the UPGMA algorithm. Colony morphologies found on YGCBA were type A (light blue, matt, rough colony surface) and type B (shiny bright blue colony surface surrounded by a white edge). Asterisks indicate isolates and starters from 2004.

starters, PFGE analyses were performed on a total of 37 surface isolates of batches 1 to 4 and nine representatives of the bacterial smear starters that had been selected as representatives of different clusters obtained in similarity analyses based on FTIR spectra. Initially, digestion of chromosomal DNA was performed using the restriction enzymes AscI, SpeI, and XbaI, of which AscI and

SpeI were considered to be most suitable for *Arthrobacter* spp. and *Brevibacterium* spp., respectively. For each enzyme, two profiles were obtained within the surface isolates which corresponded to *A. arilaitensis* and *B. aurantiacum*, as identified by 16S rRNA gene sequence analysis. Their profiles were different from those of the smear starters (Fig. 6) and were assigned to other strains of the same two species.

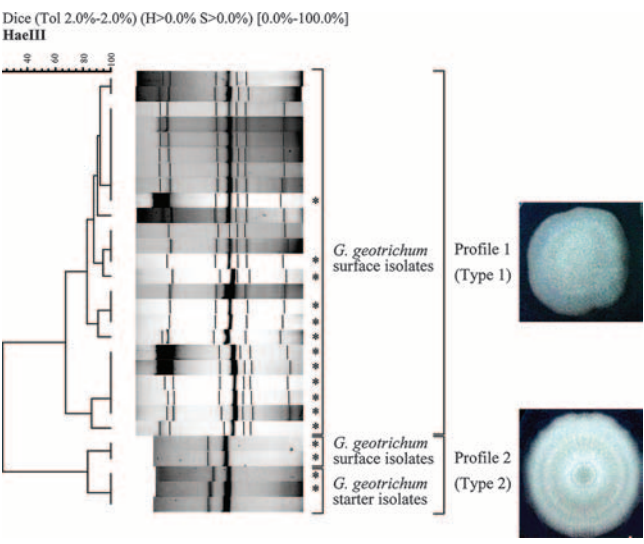Dice (Tol 2.0%-2.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
HaeIII

FIG. 4. Mitochondrial DNA RFLP profiles of *G. geotrichum* surface isolates as well as *G. geotrichum* starters. Purified DNA was digested with HaeIII. Similarities among band patterns were calculated using the Dice similarity coefficient and the UPGMA algorithm. Colony morphologies found on YGCBA were type 1 (turquoise- to blue-colored colony surface) and type 2 (light green and fluffy colony surface). Asterisks indicate isolates and starters from 2004.
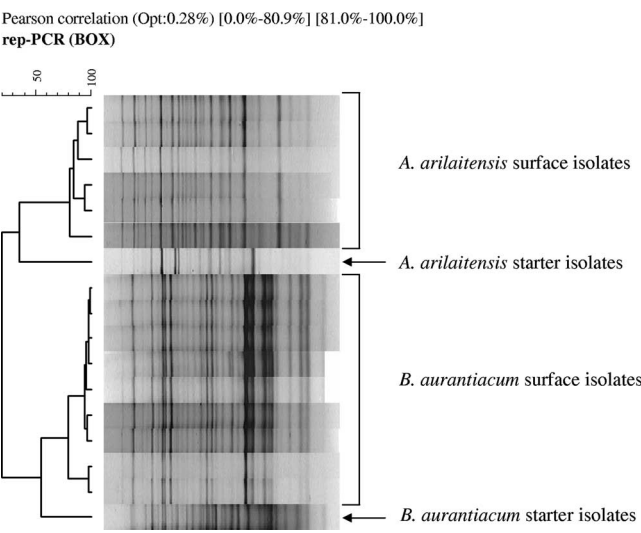
Pearson correlation (Opt:0.28%) [0.0%-80.9%] [81.0%-100.0%]
rep-PCR (BOX)

FIG. 5. Patterns obtained by *rep*-PCR using BOXA1R primers corresponding to *A. arilaitensis* surface isolates and *A. arilaitensis* starters as well as *B. aurantiacum* surface isolates and *B. aurantiacum* starters. The dendrogram was obtained after numerical analysis of *rep*-PCR patterns. Similarities among band patterns were calculated using Pearson's similarity coefficient and the UPGMA algorithm.
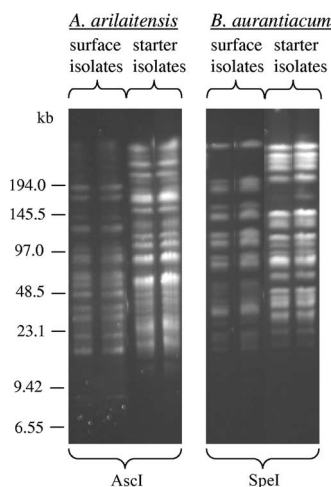
FIG. 6. PFGE band patterns of *A. arilaitensis* surface isolates and *A. arilaitensis* starters as well as *B. aurantiacum* surface isolates and *B. aurantiacum* starters. Results represent restriction digests of chromosomal DNA from *A. arilaitensis* surface isolates and starters with AscI and restriction digests of chromosomal DNA from *B. aurantiacum* surface isolates and starters with SpeI. Each pattern is shown for two isolates. A low-range PFG marker (New England Biolabs, Inc.) is shown.

DISCUSSION

Typing of microorganisms by FTIR spectroscopy. Phenotypic and genotypic methods yielded congruent results concerning the incidence of the yeast and bacterial starters on the cheese surface of the eight batches of Limburger cheese investigated. Representatives of the four starter strains could be clearly distinguished from the respective surface isolates by all methods. Since the same smear starter culture was applied during the entire examination period and since the culture proved to be very stable over time, as demonstrated by the results from 2002 and 2004, no further isolations of the starters were performed in 2006. The isolates of batches 5 to 8 were probed for the presence of the starters by FTIR spectroscopy. FTIR spectra are whole-cell fingerprints representing the total biochemical composition of the individual isolate (17). Microevolutionary changes of cellular characteristics cause variations in the spectra, resulting in several subclusters within one species (20, 27). In some cases, FTIR spectra of different FTIR clusters could be assigned to the same molecular type (Fig. 2), implying that FTIR spectroscopy may be more sensitive than BOX-PCR and PFGE. The present report is the first to demonstrate the suitability of FTIR spectroscopy for typing of coryneform bacteria below the species level. A clinical study has also shown that FTIR spectroscopy can be used to identify *Candida albicans* strains (32), a finding which was comprehensively confirmed for nonpathogenic yeast species in the present study.

Dominance of the recently described species *A. arilaitensis* or *B. aurantiacum*. The coryneform bacteria isolated from the Limburger cheese samples, as well as those from the smear starter culture, were identified as either *A. arilaitensis* or *B. aurantiacum*, two recently described species (15, 18). Both species are typical for cheese isolates (13–15, 18, 24, 29). Previously, the isolates examined in the present study had been attributed to *Arthrobacter nicotianae* and *Brevibacterium linens*,

respectively. It is not known whether *Arthrobacter* or *Brevibacterium* strains isolated from other red smear cheeses should also be reclassified as *A. arilaitensis* or *B. aurantiacum* as well.

Low biodiversity and high temporal stability of the resident ripening microorganisms. The biodiversity of the Limburger cheese flora proved to be low compared to the results of other studies (21, 22, 29, 31, 34, 36). However, yeast or bacterial floras of lower complexity have also been reported in other studies (22, 24). The two yeast species isolated are very common on Limburger cheese and on smeared cheeses in general (1, 12, 21, 31, 34). Bockelmann (1) reported identification of additional yeast species on Limburger, Romadur, and similar cheese varieties, but their numbers were quite low. However, no *Corynebacterium* species which are considered to be the dominant bacteria on surfaces of smeared cheeses (3, 8, 22, 23, 26) were detected. The low diversity might be caused by the extremely competitive resident microbes. It is well known that the house microflora of a manufacturer significantly influences the microbial ripening consortia of smear-ripened cheeses (1, 25). The major components of the in-house microflora appeared extremely stable over the entire examination period. Although the dairy built new ripening cellars which were disinfected once per week, the nonstarter (resident) strains were able to persist and to successfully establish on the cheese surface.

Commercial starter cultures inoculated into the cheese milk do not establish successfully in resident consortia. The commercial smear starter culture used contained one strain each of *B. aurantiacum* and *A. arilaitensis* and one strain each of *D. hansenii* and *G. geotrichum*. None of the bacterial or yeast smear starters was able to compete significantly, and in a stable fashion, within the cheese surface consortia, although the respective nonstarter strains belonged to the same species as the starter strains. The *D. hansenii* starter strain occurred in significant numbers on cheeses smeared the first time in batches 4, 5, and 7, but the numbers decreased strongly in further ripening stages. In all the other batches and ripening stages it was found in low numbers. The *G. geotrichum* starter strain established especially throughout the ripening of batch 7, directly after the relocation to the new ripening room. However, numbers in batch 8 were comparable to those of the other batches. Cheese surfaces of batches 1 to 8 were colonized by strains of *A. arilaitensis* and *B. aurantiacum* that were different from both starter strains (see, e.g., Fig. 2).

Both yeast starter strains remained the same over the examination period. Interestingly, the yeast strains established differently on cheese surfaces of batches 4 to 8 compared to those of the other batches (Table 1). The flora composition of these five batches has changed significantly: (i) the *D. hansenii* nonstarter strain which dominated in batches 1 to 3 was not isolated from the cheese surfaces; (ii) no further *D. hansenii* strain was found among the starter strains; (iii) the *G. geotrichum* starter strain was isolated, though in small numbers (with the exception of batch 7 and the day 3 time point in batch 6); and (iv) the *G. geotrichum* nonstarter strain which persisted over the entire examination period dominated the floras of all cheeses investigated from batches 4 to 8 except after the first smearing and during the last two samplings in batch 8. It seems that the environmental conditions had changed from batch 4 on in a way that boosted in particular the numbers of the

nonstarter *G. geotrichum* strain and repressed, on the other hand, the numbers of the nonstarter *D. hansenii* strain which had dominated the first three batches. A change in the yeast flora was also noticed by Feurer et al. (14) in studies of a French soft red smear cheese, but those authors did not differentiate at the strain level. Petersen et al. (28) observed a succession of *D. hansenii* strains appearing within the first days of ripening of a Danbo-type cheese. After 3 days, however, only one strain dominated.

The present findings point out the influence of the adventitious resident microflora in cheese ripening. In studies by Mounier et al. (25) of sources of the adventitious microflora of an Irish smear-ripened cheese, the dairy environment and the skin of the dairy personnel proved to be important. To avoid contaminations with undesirable or even pathogenic organisms such as *Listeria monocytogenes* or *Staphylococcus aureus* (34), the development of a defined smear culture and its systematic application is urgent in order to have a low-risk alternative to the old-young smearing technique. For this purpose, defined and optimized smear starter cultures have to be composed of strains that are able to compete with well-adapted in-house consortia in order to establish successfully on the cheese surface (1, 2). Certain strains may not be suitable for smear cheese ripening, while others have been used successfully in defined cultures of some cheese types (1, 4, 6). Furthermore, a commercial culture has to be applied in an optimized way. In the present study the method of application to the cheese milk might have exerted a significant influence on the failure of the commercial culture to establish successfully on the cheese surface. As a result of inoculating the cheese milk, most of the smear starter organisms are located inside the cheese body and not on the cheese surface. So it is probable that a much smaller portion of the ripening organisms than that specified per dose by the culture supplier gets the chance to colonize the cheese surface. As a further explanation of why the bacterial smear starter strains specifically could not be reisolated from the cheese surface, it might be assumed that the ripening organisms, in particular the acid-sensitive coryneform bacteria, are subject to lactic acid stress when they are simultaneously inoculated into the cheese milk with the lactic acid starter culture. Brennan et al. (8) reported an inhibitory effect of staphylococci and coryneforms for a starter culture strain which could not be recovered from the cheese surface at any time during ripening. In the present study, potential causes responsible for the disappearance of the starter strains were not further examined. Since the present study was a case study in a large dairy where cheeses are produced in a specific way for sale, it was not possible to change the production process to test whether the culture application has any influence on the starter strain establishment. Future studies using a model for cheese production should address this problem extensively.

To efficiently use this commercial culture in a dairy it should be added to the smear water and also to the cheese brine, as suggested by Bockelmann et al. (6). Inoculating the smear water alone might also be ineffective, as demonstrated by Mounier et al. (24), who examined the microfloras of four Irish farmhouse smear-ripened cheeses at the midstage of ripening. Three of these cheeses were smeared with commercial smear starters; none of commercial smear starter strains were reisolated from the cheese surfaces. On a Danbo-type cheese, the *D. hansenii* starter strain additionally used in the old-young smearing water was detected in a maximum fraction number of 30% during the early stages of ripening. Further studies of the same cheese after 3 days of ripening showed its presence on only 2 out of 16 cheeses, but only in small numbers (28). Another interesting approach would be to see whether the nonstarter surface isolates could be effective as starters themselves instead of using the commercial strains in the same dairy or under different production conditions.

Concluding remarks. The present report demonstrates that the starter strains do not outcompete the resident microbiota. It cannot be decided whether this is due to the application of the smear starters to the cheese milk or to the competitive potential of the resident microfloras or to both of these factors. Based on the data presented here, it may be questioned whether addition of the starter culture for producing this South German Limburger red smear cheese is actually necessary. The Limburger cheese investigated in this study may well be produced at a reduced cost without adding any commercial starter strains to the cheese milk. Back-slopping processes, such as the old-young smearing technique, are known to enable the development of a potentially strongly adapted in-house ripening flora, which often significantly contributes to the sensory properties (3). Furthermore, a properly established microflora can serve as an effective protection against spoilage and pathogenic bacteria via competition. Therefore, a traditional old-young smearing technique associated with an adequate hygienic concept might be an appropriate option for the German dairy to produce a safe high-quality cheese. It would also be an interesting alternative to directly use the house microflora as a smear starter culture to boost its positive effect. Nevertheless, since undesired organisms such as *L. monocytogenes* can become part of the in-house microbial ripening consortium and persist in the dairy by this back-slopping cycle, the long-term aim should be to completely avoid the old-young smearing process and, instead, to produce cheese merely by using optimized, defined ripening starter cultures (1, 4, 6) applied in an effective way as soon as such starters become available. Since food safety has top priority, the development of suitable, defined surface starter cultures is urgent.

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